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PRINCIPAL INVESTIGATOR: Yi-Hsin Liu, Ph.D.

CONTRACTING ORGANIZATION: University of Southern California
Los Angeles, California 90033

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Yi-Hsin Liu, Ph.D.

7. PERFORMING ORGANIZATION NAME(S) AND ADDRESS(ES)
University of Southern California
Los Angeles, California 90033

E-Mail: yhliu@hsc.usc.edu

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13. ABSTRACT *(Maximum 200 Words)*

The purpose of this study is to determine the role of a transcriptional factor, Msx2, in regulating branching events in the development of the mouse mammary gland. To define the function of Msx2 gene in mammary gland development, we utilized targeted null mutants and transgenic animals. Besides a defect in elongation and branching in the null mutant mammary glands, we observed elongation and branching abnormalities associated with heterozygous null mutants which indicates that mammary glands development is sensitive to Msx2 gene dosage. This is the first demonstration that mammary gland branching and elongation can be modulated by the level of gene expression of a homeodomain transcriptional factor.

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Table of Contents

INTRODUCTION:

Breast cancer is the second most common form of malignancy and the second leading cause of fatality among women who have cancer. And yet, the underlying mechanisms that lead to the formation of mammary tumors remain unclear. In this study, we utilized genetically engineered mouse models to investigate the signaling mechanism that controls normal breast development with the premise that tumorogenesis of the breast is a recapitulation of normal developmental processes with the exception that it is unregulated. We have limited our study to examine in greater detail the phenotypic changes in mouse mammary gland development that occurred due to the removal or overexpression of a transcriptional factor, Msx2.

BODY:

The mammary gland undergoes a series of well-coordinated morphogenetic processes prior to lactation (Hennighausen and Robinson, 2001). In the mouse, the anlage of mammary epithelium at birth is a small, branched structure connected to the nipple. With the onset of puberty at around four weeks of age, there is rapid proliferation within the terminal end-buds (TEBs) which results in the elongation of the ducts into the fat pad and subsequent elaboration of the structure as a consequence of tertiary side-branches. Development of the anlage into a functional mammary gland in female mice represents a well-orchestrated sets of gene actions of different endocrine and local factors during each phase of mammary growth. During the initial phases of mammary development, reciprocal signaling between the epithelium and the underlying mesenchyme results in the formation of the mammary bud. Cell proliferation leads to the formation of the primary sprout that invades the fat pad. Msx2 together with other factors, both growth factors, receptors and transcriptional factors included, are expressed by the mesenchyme surrounding the invaginating epithelial mammary bud (Satokata et al., 2000). In the Msx2 null mutants, mammary gland development was shown to arrest at the initial stage of mammary gland development (Satokata et al., 2000). It was shown that parathyroid hormone related peptide receptor (PPR1) gene expression was down-regulated in the Msx2 null mutant mammary buds (Satokata et al., 2000). The ligand of PPR1, PTHrP is expressed in mammary epithelium, and the signal is received by the surrounding mesenchymeal cells that express PPR1. Interruption of PTHrP signaling was shown to cause developmental arrest of the mammary gland primordium before elongation of the bud into a primary sprout (Wysolmerski et al., 1998). Ectopic expression of PTHrP in the epidermis under the control of K14 promoter caused the adjacent dermal cells to assume characteristics of primary mammary mesenchyme and differentiate as nipple cells (Foley et al., 2001). In other words, PTHrP is the first signaling molecule known to be made by the embryonic mammary epithelial cells that influence the cell fate decisions of the underlying mesenchyme.

Following the embryonic and prepubertal stages, further development of the mammary gland becomes hormone-dependent. Although a large number of studies have suggested the requirement for estrogen and progesterone during puberty phase of mammary gland development, how these hormones regulate other genetic components remains unclear. Hormonal actions on post-pubertal mammary gland development were initially revealed by hormone depletion studies through endocrine ablations and defined hormone reconstitution studies. Gene targeting of hormonal receptors has provided further genetic evidence of hormonal regulation of mammary gland development. In estrogen receptor alpha null mutant, ductal development during puberty is severely curtailed. The phenotype was complicated by the fact that estrogen signaling has systemic effects due to broad expression of ER receptors in ovaries, uterus and pituitary besides mammary gland. Estrogen receptor alpha knockout resulted in defective pituitary function and non-functional corpus luteum and consequently reduced levels of prolactin and progesterone synthesis (Bocchinfuso et al., 2000).

In the original proposal, we proposed to integrate Msx2 into the signaling pathways of estrogen and PTHrP. However, during the analysis of Msx2 null and Msx2 transgenic mammary phenotypes, we realized that our model requires further refinement. When the proposal was submitted and funded, we had a very limited picture of mammary development in the Msx2 null mutant and Msx2 transgenic animals. After we got more into the phenotypic characterization, we realized that we need to perform a detailed systematic characterization of mammary gland development in the Msx2 null and transgenic animals before engaging into a laborious genetic analysis of crosses involving Msx2 mutants and estrogen receptor targeted null mutants and

PTHrP receptor transgenics. Our study of mammary gland development in the Msx2 null mutants and Msx2 transgenic animals should provide newer insight into how gene dosage might influence elongation and branching of developing mammary gland.

Msx2 is one of three related mammalian genes, *Msx1*, *Msx2* and *Msx*3, that constitute the *msh* gene family (Davidson, 1995). It encodes a homeodomain transcriptional factor that is known to play a critical role in regulating calvarial bone and suture development, mammary gland genesis and hair follicle formation (Jabs, *et al.*, 1993; Liu *et al.*, 1995, 1999; Jiang *et al.*, 1999; Satokata *et al.*, 2000; Wilkie *et al.*, 2000). Null mutations in the *Msx2* gene have been shown to arrest hair follicle and mammary gland development in the mouse and to delay and prevent ossification of the parietal foramina in both mouse and man (Satokata *et al.*, 2000; Wilkie *et al.*, 2000). Furthermore, overexpression of the *Msx2* gene in transgenic animals resulted in hypokeratosis of the skin and enhancement of ossification of skull bones as a result of enhanced proliferation of osteoprogenitors (Liu *et al.*, 1995; Jiang *et al.*, 1999; Liu *et al.*, 1999). A hypermorphic mutation in *MSX2* is the cause of Boston type craniosynostosis (Jabs *et al.*, 1993).

In the original paper describing the Msx2 null mouse mutant, Satokata *et al.* (2000) stated that "one-third of *Msx2*-mutant females exhibited abnormal mammary development. In these mutants, the mammary epithelium arrested at the mammary sprout stage of embryonic development. The mesenchymal expression of *Msx2* at this developmental stage suggests that *Msx2* acts to promote branching morphogenesis of the mammary epithelium.... In addition, all male and female *Msx1*, *Msx2* double mutants show a failure of mammary epithelial invagination, resulting in subsequent regression." After obtaining Msx2 null mutant animals, we confirmed the observation that in Msx2 null mutant females mammary gland development arrested at the initial sprout stage (Figure 1). The phenotype was highly variable among null mutant animals ranging from minimal sprouting without TEBs as shown in figure 1C to extensive sprouting with large TEBs as shown in figure 1B.

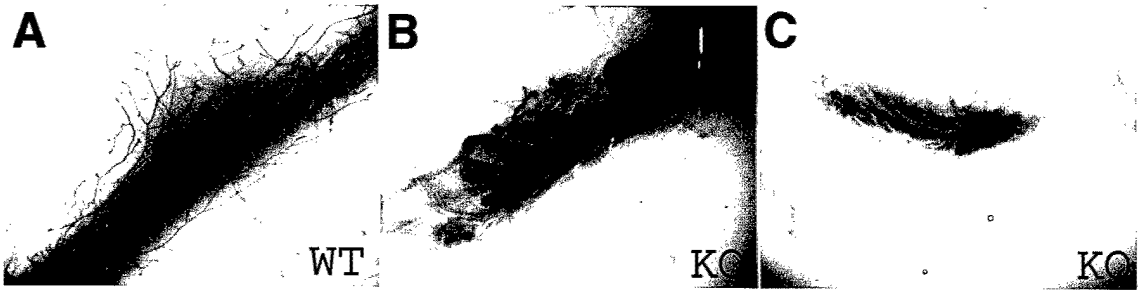


Figure 1: Variable phenotypes of post-pubertal mammary glands in Msx2 homozygous null mutant animals. (A) Whole-mount carmine stained mammary gland from a 6 week old wildtype female. (B) Mammary gland from a 6 week old Msx2 knockout female. The extent of sprouting is similar to a wildtype mammary gland at 4 weeks. (C) Mammary gland from another 6 week old Msx2 knockout female. Very little development of the mammary gland is observed.

After observing the large variability of mammary gland phenotypes in the Msx2 null mutants, we immediately saw possible difficulty in performing phenotypic analysis if we proceed to cross Msx2 null mutants to ER alpha targeted mutants or PPR1 transgenic animals. However, while doing phenotype analysis of the Msx2 null mutants, we noticed a phenotypic difference between the wild-type mammary gland and mammary glands from Msx2 heterozygous knockout animals. We focused all our effects to precisely define the phenotype in the heterozygotes Msx2 knockout animals and Msx2 transgenic animals. Work is still ongoing to investigate the involvement of pituitary/thalamus axis and endocrine signaling.

In four-week old animals, the ductal length of developing mammary glands was indistinguishable between wildtype and Msx2 heterozygous females ($p > 0.05$) (Figure 2 and 3). Interestingly, in the Msx2 transgenic animals, ductal elongation was severely retarded ($p < 0.001$) (Figure 2 and 3). In wildtype mammary glands, the average distance between the nipple and the terminal end buds was 6.07+/-0.55mm (n=5) whereas in

the heterzygotes, the mean distance was 5.53+/-0.67mm (n=7) (Figure 3). However, ductal elongation was curtailed in Msx2 transgenic mammary glands which showed an average ductal length of 2.88+/-0.57mm (n=7) (Figure 3).

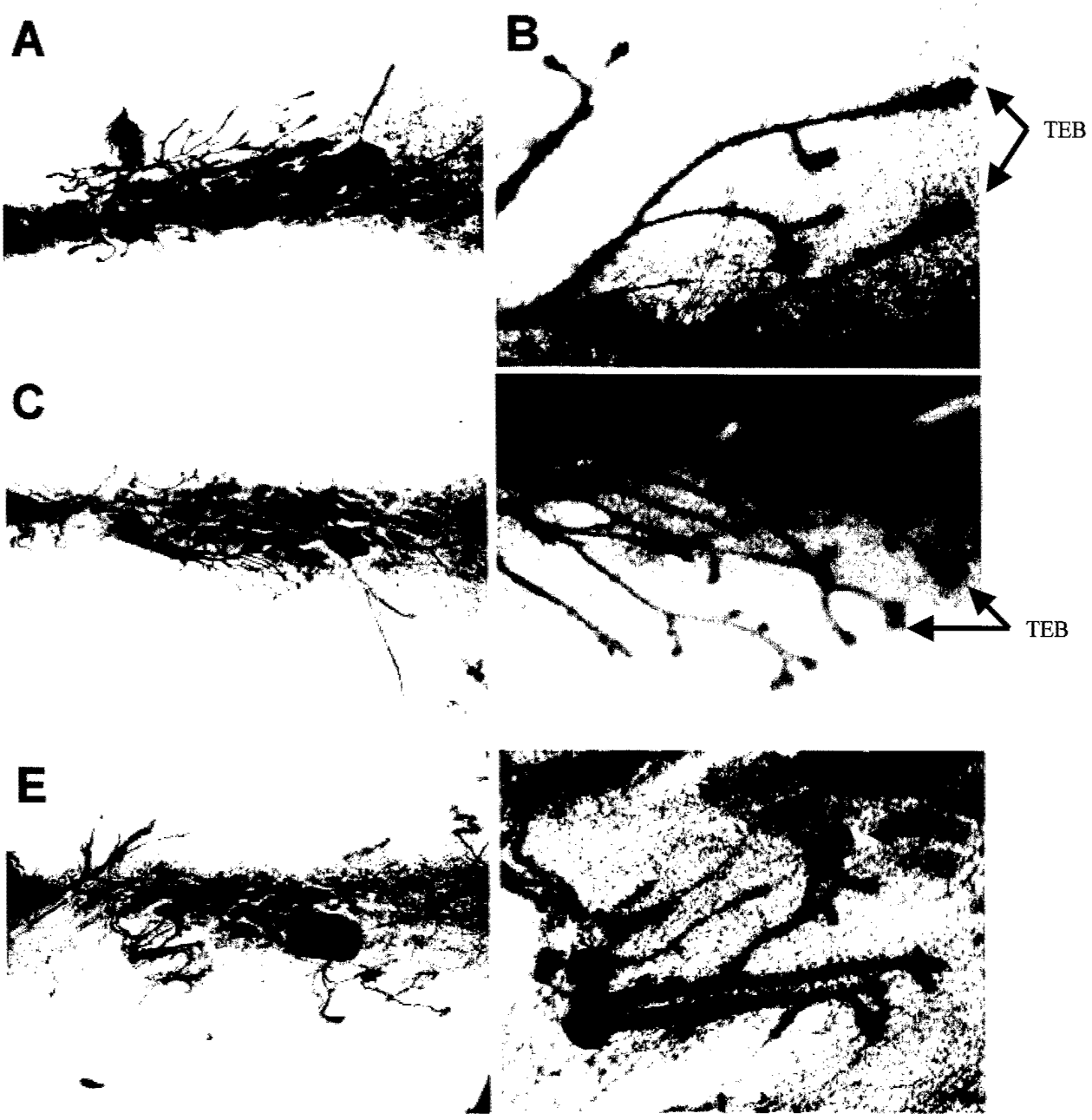


Figure 2. Wholemounts of mammary glands from 4-week old females. (A) Mammary gland from a wildtype female. (B) Magnified view of the mammary gland in (A). (C) Mammary gland from a Msx2 heterozygous female. (D) Magnified view of (C). (E) Mammary fat pad from a Msx2 transgenic female. Branching and ductal elongation are severely retarded. (F) Magnified view of (E).

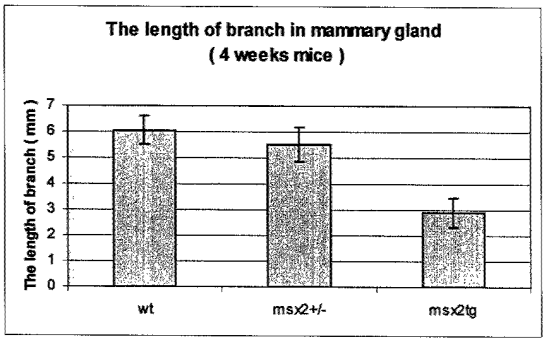


Figure 3. Summary graph of mammary primary ductal length

At six weeks, the primary ducts in the wildtype mammary gland had extended beyond the lymph node with extensive secondary and tertiary branches (Figure 4). The average ductal length of wildtype mammary glands is 17.44 +/- 2.47 mm (n=19) (Figure 5). On the contrary, ductal elongation in the Msx2 heterozygotes null animals was severely curtailed (Figure 4). The average ductal length in these animals was 9.76 +/- 2.41 mm (n=27), almost half the size of the wildtype counterpart. No significant gain in ductal branching and elongation when compared with mammary glands at four weeks of age. Unexpectedly, ductal elongation and branching were significantly enhanced in Msx2 transgenic mammary glands (Figure 4). The average ductal length in Msx2 transgenic mammary glands reached 12.78 +/- 1.51 mm (n=13) although the phenotypic difference remained between the wildtype and Msx2 transgenic mammary glands (Figure 5). The total number

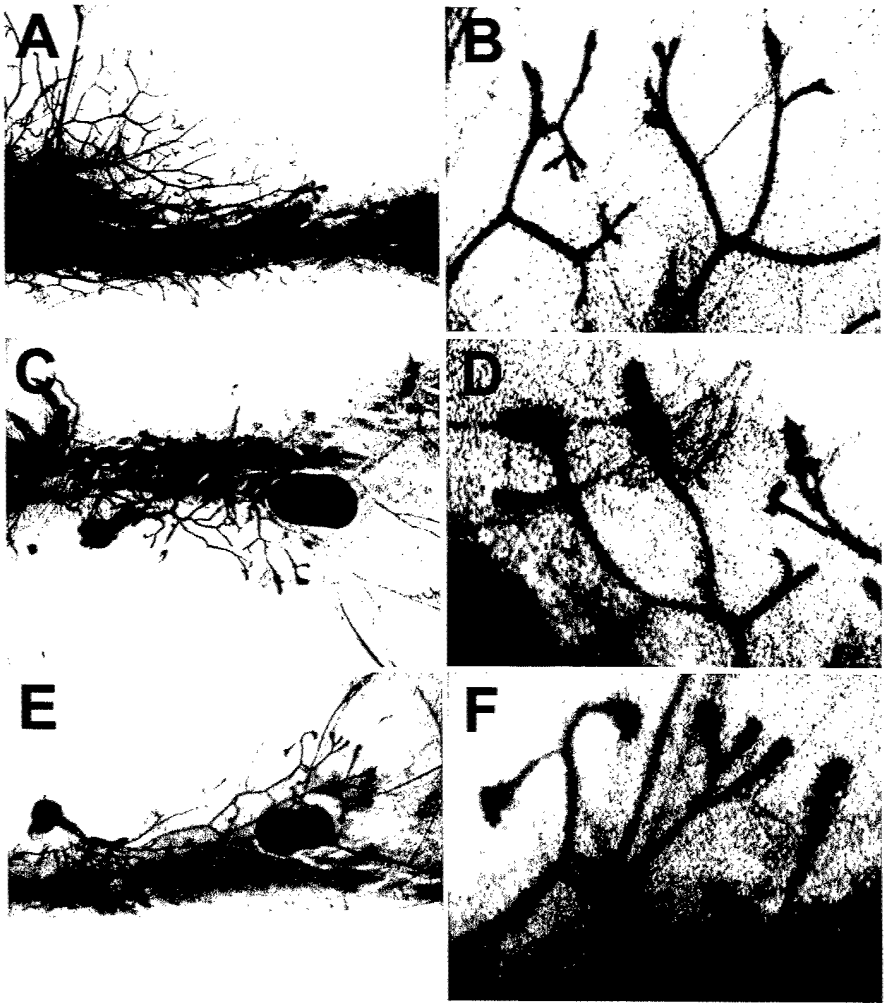


Figure 4. Wholemounts of mammary glands from 6-week old females. (A) Mammary gland from a wildtype female. Extensive branching allowed spreading throughout the fat pad and primary ducts had grown beyond the lymph node. (B) Magnified view of the mammary gland in (A) showing secondary and tertiary branches. (C) Mammary gland from a Msx2 heterozygous female. Both ductal elongation and branching were inhibited. (D) Magnified view of (C) showing terminal end buds. (E) Mammary fat pad from a Msx2 transgenic female. Branching and ductal elongation were retarded. (F) Magnified view of terminal end buds in (E).

of primary, secondary and tertiary branches were significantly different among wildtype, Msx2 heterozygotes and Msx2 transgenic mammary glands. The average total number of branches in wildtype mammary gland

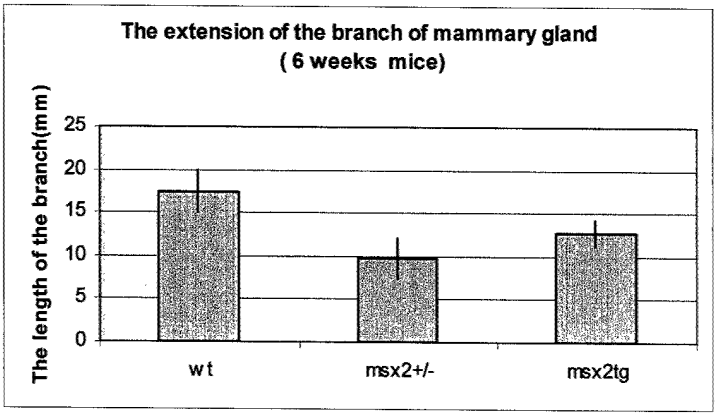


Figure 5. Summary graph of mammary primary ductal length

was approximately 29, whereas in Msx2 heterozygotes, the average number of branches was about 10 versus 15 in Msx2 transgenic animals (Figure 6).

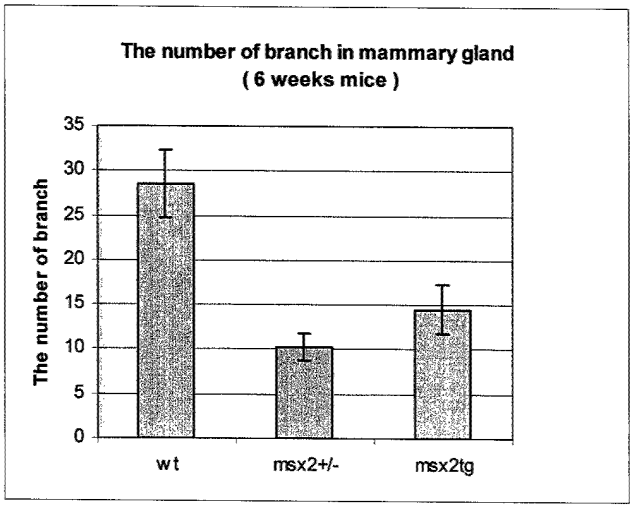


Figure 6. Quantitative analysis of branching number in 6-week old virgin wildtype, Msx2 heterozygotes, and Msx2 transgenic animals.

Unexpectedly, by 12 weeks, ductal elongation in the Msx2 heterozygous knockout and Msx2 transgenic animals was indistinguishable from that in the wild type mammary glands (Figure 7). However, branching and alveolar lobular structures remained defective. There were far more secondary and tertiary branches in the wildtype mammary glands as depicted in Figure 7A. Transgenic mammary glands were lagging far behind in their tertiary branches (lobular sprouts) (Figure 7) in comparison to that of wildtype and heterozygous knockout animals.

In order to understand the cellular mechanism underlying these developmental defects, we decided to examine the rate of cell proliferation in terminal end buds which are known to be involved in ductal elongation. So far, we have performed BrdU labeling in 6-week old virgin mammary glands. The result may provide a preliminary explanation as to why mammary glands of Msx2 heterozgous null animals and Msx2 transgenic animals were able to recover from ductal elongation deficiencies. In TEBs of Msx2+/- mammary glands, more than 27% of cells were actively dividing versus 7% of epithelial cells in wildtype mammary glands. A moderate increase in proliferating cells in TEBs was observed in Msx2 transgenic mammary glands (13% of cells were actively dividing) (Figure 8). Further analysis of proliferation and apoptosis at 4, 5, 7 and 8 week old mammary glands is required to understand the correlation between cell proliferation and observed phenotype.

To address the original hypothesis that estrogen signal transduction pathway is involved in the mammary gland defects of Msx2 null and Msx2 transgenic animals. We examined alterations in protein expression of the Estrogen Receptor alpha.(ERα) by performing Immunohistochemistry on paraffin sections of

embedded mammary glands. We did not observe a significant change in ERalpha expression in TEBs (Figure 9).

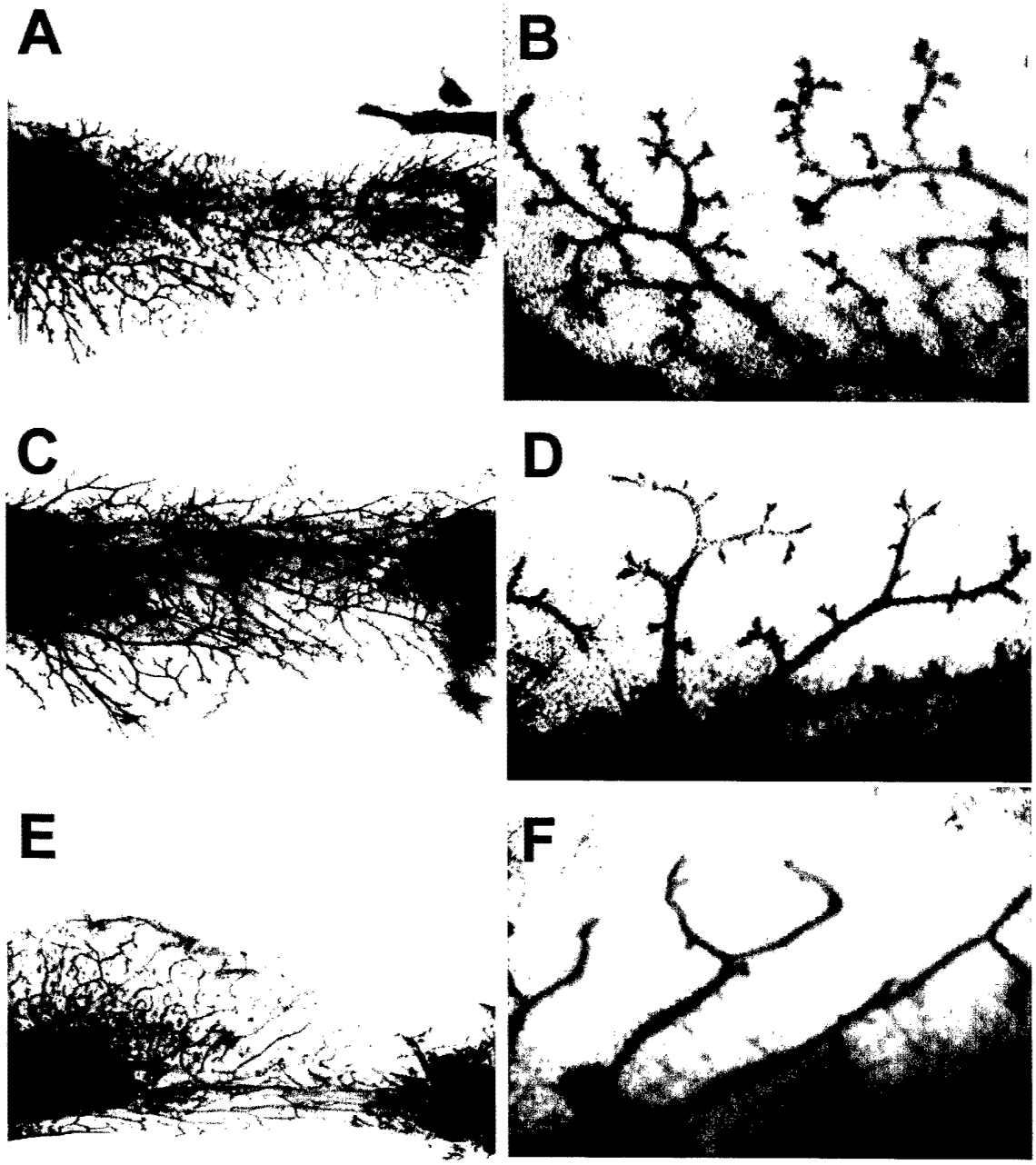


Figure 7 Wholemounts of mammary glands from 12-week old females. (A) Mammary gland from a wildtype female. Extensive branching allowed spreading throughout the fat pad and primary ducts had grown beyond the lymph node and reached the boundary of the fat pad. (B) Magnified view of the mammary gland in (A) showing secondary and tertiary branches and lobular sprouts. (C) Mammary gland from a Msx2 heterozygous female. Primary ducts have reached the boundary of the fat pad. (D) Magnified view of (C) showing terminal sprouts. (E) Mammary fat pad from a Msx2 transgenic female. Less elaboration of terminal branches was observed. (F) Magnified view of terminal branches in (E). Notice the lacking of terminal sprouts.

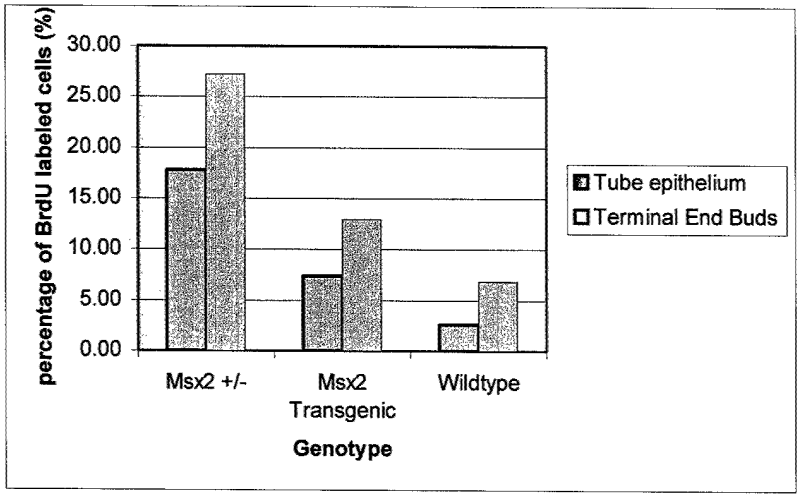
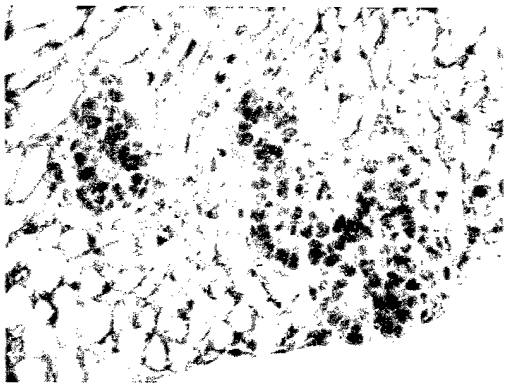


Figure 8: Rate of mammary epithelial cell proliferation in Msx2 +/- and Msx2 transgenic animals were accelerated. Six-week old females were given BrdU. Two hours later, mammary glands were dissected out and processed for paraffin embedment. BrdU labeled nuclei were visualized by performing Immunohistochemistry using mouse monoclonal antibody for BrdU. Percentage of BrdU-labeled cells were calculated by taking the ratio between BrdU-immune-reactive cells and total number of epithelial cells. Tube epithelium represents cells lining the ductal wall.



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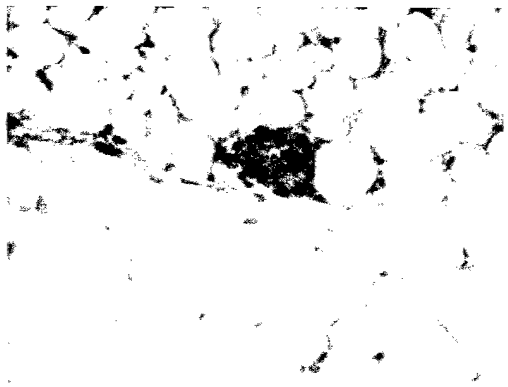
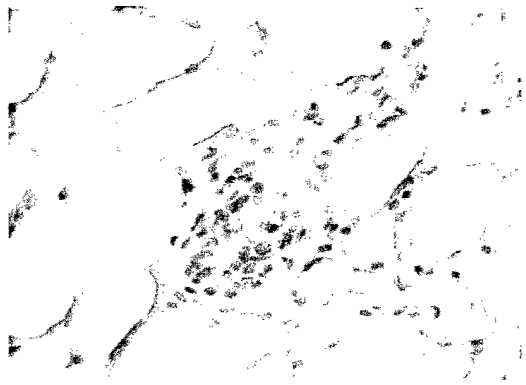


msx2+/-



msx2tg

Figure 9: Immunostaining of ER-alpha in TEBs of six-week old mammary glands.

Key Research Accomplishments

Msx2 haploinsufficiency can lead to a delay in mammary gland elongation and inhibition of lateral branching.

Overexpression of Msx2 also antagonizes mammary gland elongation and lateral branching.

This is the first report that mammary gland development can be perturbed by a Homeobox gene by simply altering gene dosage.

Msx2 functions downstream of Estrogen receptor alpha signaling pathway.

Reportable Outcomes

A manuscript describing the mammary gland phenotype of Msx2 knock-out and Msx2 transgenic animals is being prepared.

CONCLUSIONS: In this study, we have demonstrated that haploinsufficiency of Msx2 can lead to developmental defects in the mouse mammary gland early in puberty. The primary defects are a delay in ductal elongation and lateral branching. In addition to its involvement in the induction of mammary anlage, Msx2 is a key regulator of mammary elongation and branching during puberty. **Since the expression of estrogen receptor alpha was not altered in Msx2 heterozygous null and transgenic animals together with results from other studies showing induction of Msx2 gene expression by estrogen (Phippard et al., 1996; Friedmann and Daniel, 1996), we conclude that Msx2 functions downstream of estrogen receptor alpha signaling cascade. Msx2 may serve as a therapeutic target that may partially block the actions of estrogen in the mammary gland. Further studies are needed to understand whether Msx2 is a direct target or an indirect target gene of estrogen and what are the downstream targets of Msx2.**

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List of Personnel involved in this project:

Following personnel were involved in this project at different stages. Shaoyun Jiang did not receive compensation from this grant.

Fengfeng Zheng	Research Assistant
Min Li	Research Associate
Lin Guo	Research Assistant
Shaoyun Jiang	Research Associate

APPENDICES:

NONE